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(54) Title: VACCINE FOR MYSTERY SWINE DISEASE AND METHOD FOR DIAGNOSIS THEREOF

(57) Abstract

The invention includes a vaccine and sera for treatment of Mystery Swine Disease (MSD), a method for producing the vaccine, and methods for diagnosis of MSD. The vaccine is derived from an MSD infectious agent that is isolated pursuant to the methods disclosed in the present invention. The serum for treatment of infective swine is derived from the blood plasma of a non-swine mammal pretreated with the MSD infectious agent. The serum contains mammalian antibodies which are effective in treating MSD.

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# VACCINE FOR MYSTERY SWINE DISEASE AND METHOD FOR DIAGNOSIS THEREOF

## BACKGROUND OF THE INVENTION

Since 1987, the swine-producing industry has been subjected to a devastating epidemic of an unknown disease, often referred to as "Mystery Swine Disease" (MSD) because researchers have been unable to identify the causative agent. MSD has affected hundreds of thousands of swine throughout North America and Europe. Once one pig is infected with MSD, that one pig can spread the MSD to an entire herd within three to seven days. From 1987 to 1991, the swine industry has lost millions of dollars in revenue as a result of MSD. A recent study estimates that MSD causes a financial loss between $250 and $500 per inventoried sow.

MSD causes multiple symptoms in swine. The first symptom of MSD in a breeding herd of swine is usually anorexia and mild pyrexia. In addition, the herd animals may exhibit bluish discolorations in their skin, especially in their ears, teats, snout, and the frontal portions of their necks and shoulders. The affected skin may become irreparably damaged. However, the most devastating symptom of MSD is the reproductive failure that occurs in a breeding herd of swine. MSD causes sows to bear stillborn piglets, undersized-weak piglets with respiratory distress, or piglets which die before they are weaned. Other reproductive symptoms caused by MSD include early farrowing of piglets, a decrease in conception rates, failure in some sows to cycle, and a reduction in the total number of piglets found in a litter. It has been estimated that the number of pigs lost from reproductive failure is about 10 to 15 percent of the annual production of pigs.

Research has been directed toward isolating the causative agent of MSD. A number of potential bacterial pathogens have been isolated. However, the types of potential bacterial pathogens have varied between swine-

producing farms. Viral investigation has included fluorescent antibody examination, electron microscopic investigation, and serology. These methods have failed to locate the causative agent of MSD. As a result, no one has yet developed a vaccine which can be used to treat MSD in the swine population.

Therefore, it is an objective of the invention to provide a vaccine and sera which, when administered to a breeding swine herd, will reduce the presence of MSD in their population. Another object is to provide a method of treating a population of swine with the vaccine to eradicate MSD from the swine population. Yet another object is to provide a method for diagnosis of MSD.

## SUMMARY OF THE INVENTION

These and other objects are achieved by the present invention which is directed to a vaccine and sera for prevention and treatment of mystery swine disease and to a method for its diagnosis in swine.

The vaccine is derived from an infectious agent that will infect swine with mystery swine disease (MSD). The infectious agent is obtained from an inoculum of processed tissue of swine infected with the disease, preferably lung tissue. Preferably, the infectious agent is the product of an *in vitro* swine cell culture infected with the inoculum of the infected swine tissue. Preferably, the inoculum contains biological particles no greater than about 1.0 microns in size, more preferably 0.5 microns, most preferably no greater than 0.2 microns. It is also preferable that the inoculum has been neutralized with antibodies to common swine diseases.

The serum for treatment of infected swine carries mammalian antibodies to the MSD. It is obtained from the blood plasma of a non-swine mammal pretreated with the above-described infectious agent.

Alternatively, the serum is formulated from monoclonal antibodies to MSD produced by hybridoma methods.

The method for diagnosis of MSD is based upon the use of immunospecific antibodies for MSD. The method calls for combination of a filtered homogenate of a lung biopsy sample or other tissue sample and the immunospecific antibodies followed by application of a known detection technique for the conjugate formed by this combination. Immobilization or precipitation of the conjugate and application of such detection techniques as ELISA, RIA, Southern Blot and the like will diagnose MSD.

## DETAILED DESCRIPTION OF THE INVENTION

Determination of the cause of Mystery Swine Disease (MSD) has been difficult. Until the present invention, researchers have been unsuccessful in their attempts to isolate bacteria and/or viruses from tissues of infected swine. According to the present invention, however, the isolation and growth of the infectious agent causing MSD has been achieved. The isolation is a major breakthrough and discovery. It enables the production of vaccines, antibody sera for treatment of infected swine and diagnostic methods.

The vaccine is composed of an inactivated or attenuated MSD infectious agent derived from an inoculum processed from infected swine lung tissue or other swine tissue exhibiting the characteristic lesions of MSD. A multi-step procedure is utilized in developing the MSD vaccine. The MSD infectious agent is first obtained as an inoculum by separation and isolation from infected swine tissue, preferably the lung tissue. The MSD infectious agent is then treated using known immunological techniques to form a vaccine against MSD.

The MSD infectious agent is preferably isolated as an inoculate from lung tissue of pigs which exhibit

rapid breathing due to the MSD. Such pigs are destroyed and their lung tissue removed. The lung tissue is then microscopically examined for thickened alveolar septae caused by the presence of macrophages, degenerating cells, and debris in alveolar spaces. These characteristics indicate the presence of the MSD infectious agent. Other swine tissue exhibiting lesions of this sort may be also used to isolate the MSD infectious agent.

The lung or other swine tissue is then homogenized with a pharmaceutically acceptable aqueous solution (such as physiological saline, Ringers solution and the like) such that the tissue comprises 10% weight/volume amount of the homogenate. The homogenate is then strained through micron filters in the 0.05 to 10 micron range, preferably through a series of 0.45, 0.2 and 0.1 micron filters, to produce a filtered homogenate containing the MSD infectious agent. As a result, the filtered homogenate contains biological particles having a size no greater than about 1.0 micron, preferably no greater than 0.2 to 0.1 micron. The filtered homogenate can then be mixed with Freund's incomplete adjuvant so that the production of antibodies can be stimulated upon injection into a mammal. This mixture can be used as an inoculum for development of MSD in swine or further study of the MSD infectious agent.

After obtaining a filtered homogenate containing the infectious agent, the infectious agent can be inactivated or killed by treatment of the filtered homogenate with a standard chemical inactivating agent such as an aldehyde reagent including formalin, acetaldehyde and the like, reactive acidic alcohols including cresol, phenol and the like, acids such as benzoic acid, benzene sulfonic acid and the like, lactones such as beta propiolactone and caprolactone, and activated lactams, carbodiimides and

carbonyl diheteroaromatic compounds such as carbonyl diimidazole. Irradiation such as with ultraviolet and gamma irradiation can also be used to inactivate or kill the infectious agent. Alternatively, the infectious agent can be attenuated by its repeated growth in cell culture from non-swine mammal or avian origin so that the ability of the infectious agent to virulently reproduce is lost. The details of the cell culture attenuation technique are given below.

The killed or attenuated infectious agent is then diluted to an appropriate titer by addition of an adjuvant solution for stimulation of immune response. The titration is accomplished by measurement against MSD antibody in a immunologic test such as an ELISA, RIA, FIA or enzyme substrate detection test as described below.

To produce a purified form of the infectious agent, the filtered homogenate described above can be inoculated into a series of in vitro cell preparations. Cell preparations with mammalian organ cells such as kidney, liver, heart and brain, lung, spleen, testicle, turbinate, white and red blood cells and lymph node, as well as avian embryo preparations can be used. Culture media suitable for these cell preparations include those supporting mammalian cell growth such as fetal calf serum and agar, blood infusion agar, brain-heart infusion glucose broth and agar and the like.

After inoculating the cell preparation with the filtered homogenate and growing the culture, individual clumps of cultured cells are harvested and reintroduced into sterile culture medium with cells. The culture fluid from the final culture of the series provides the purified form of the virulent infectious agent. Also, after a series of repeated harvests have been made, the culture can be grown, the culture fluid collected and the fluid used as an inoculum for a culture of a different cellular species. In this fashion, the

infective agent can be attenuated such that the culture fluid from the differing species culture provides the purified form of the attenuated infectious agent.

Polyclonal antibody sera can be produced through use of the infectious agent as an antigenic substance to raise an immune response in mammals. The culture fluid or inoculum prepared as described above can be administered with a stimulating adjuvant to a non-swine mammal such as a horse, goat, mouse or rabbit. After repeated challenge, portions of blood serum can be removed and antigenically purified using immobilized antibodies to those disease specific antibodies typically found in the serum of the bled animal. Further treatment of the semipurified serum by chromatography on for example a saccharide gel column with physiological saline and collection of proteinaceous components of molecular weight at least 10,000 provides a purified polyclonal sera for use in treatment.

Monoclonal antibody sera can be produced by the hybridoma technique. After immunization of a mouse, rat or rabbit with the cell culture fluid or inoculum as described above, the spleen of the animal can be removed and converted into a whole cell preparation. Following the method of Kohler and Milstein (Kohler et al., Nature, 256, 495-97 (1975)), the immune cells from the spleen cell preparation can be fused with myeloma cells to produce hybridomas. Culturation of the hybridomas and testing the culture fluid against the fluid or inoculum carrying the infectious agent allows isolation of the hybridoma culture producing monoclonal antibodies to the MSD infectious agent. Introduction of the hybridoma into the peritoneum of the host species will produce a peritonial growth of the hybridoma. Collection of the ascites fluid yields serum containing the monoclonal antibody to the infectious agent.

The vaccine of the present invention is capable of preventing and curing MSD infections found in the swine population. For effective prophylactic and anti-infectious use in vivo, the MSD vaccine contains killed or attenuated MSD infectious agent and may be administered alone or in combination with a pharmaceutical carrier that is compatible with swine. The vaccine may be delivered orally, parenterally, intranasally or intravenously. The amount and the choice of delivery of the vaccine is ultimately made by an attending veterinarian and is based upon the age and the weight of the infected pig. The range of a given dose is $10^3$ to $10^7$ Tissue Culture Infective Dose 50 per ml, preferably given in 1 ml to 5 ml doses. The vaccine doses should be applied over about 14 to 21 days to ensure that the pig has developed an immunity to the MSD infection.

The MSD vaccine can be administered in a variety of different dosage forms. An aqueous medium containing the killed or attenuated MSD infectious agent may be desiccated and combined with pharmaceutically acceptable inert excipients and buffering agents such as lactose, starch, calcium carbonate, sodium citrate formed into tablets, capsules and the like. These combinations may also be formed into a powder or suspended in an aqueous solution such that these powders and/or solutions can be added to animal feed or the animals' drinking water. These MSD vaccine powders or solutions can be suitably sweetened or flavored by various known agents to promote the uptake of the vaccine orally by the pig.

For purposes of parenteral administration, the killed or attenuated MSD infectious agent can be combined with pharmaceutically acceptable carrier(s) well known in the art such as saline solution, water, propylene glycol, etc. In this form, the vaccine can be parenterally applied by well known methods known in the

art of veterinary medicine. The MSD vaccine can also be administered intravenously by syringe. In this form, the MSD vaccine is combined with pharmaceutically acceptable aqueous carrier(s) such as a saline solution. The parenteral and intravenous formulations of MSD vaccine may also include emulsifying and/or suspending agents as well, together with pharmaceutically acceptable diluent to control the delivery and the dose amount of the MSD vaccine.

The method for diagnosis of MSD is carried out with the poly or monoclonal antibody sera described above. Either the antibody sera or the biopsied tissue homogenate may be immobilized by contact with a polystyrene surface or a surface of another polymer for immobilizing protein. The other of the antibody sera and homogenate is then added, incubated and the nonimmobilized material removed, for example, by washing. A labeled species specific antibody for the antibody sera is then added and the presence and quantity of label determined. The label determination indicates the presence of MSD in the tissue assayed. Typical embodiments of this method include the enzyme linked immunosorbent assay (ELISA), radioimmunoassay (RIA), immunofluorescent assay (IFA), Northern, Southern, and Western Blot immunoassay.

The following examples further illustrate specific embodiments of the invention. The examples, however, are not meant to limit the scope of the invention which has been fully characterized in the foregoing disclosure.

## EXAMPLE 1

The MSD infectious agent may be characterized by determining physiochemical properties (size, sensitivity to lipid solvents, and sensitivity to protease) by treatment of the inoculum followed by the

inoculation of gnotobiotic pigs to determine if the MSD infectious agent remains pathogenic.

Gnotobiotic pigs. Derivation and maintenance procedures for gnotobiotic pigs have been described in Benfield et al., Am. J. Vet. Res., 49, 330-36 (1988) and Collins et al., Am. J. Vet. Res., 50, 824-35 (1989). Sows can be obtained from a herd free of reproduction problems including MSD. Litters with stillborn and/or mummified fetuses should not be used.

MSD inoculum (MN90-SD76-GP2). Trachea, lung, turbinates, tonsil, liver, brain, and spleen can be collected from nursing pigs in a Minnesota swine herd spontaneously infected with MSD (Collins et al., Minnesota Swine Conference for Veterinarians, Abstract, 254-55 (1990)). A homogenate of these tissues (designated MN 89-35477) has been prepared in Hank's balanced salt solution without antibiotics and 0.5 ml can be intranasally inoculated into 3-day-old gnotobiotic piglets using a glass Nebulizer (Ted Pella Co., Redding, CA). Inoculated piglets can develop clinical signs and microscopic lesions similar to those observed in the spontaneously infected pigs. Lungs, liver, kidney, spleen, heart and brain from these gnotobiotic pigs can be collected 8 days after the original inoculation and pooled to prepare another homogenate. This second homogenate can then be inoculated one additional time in gnotobiotic pigs. Again the same tissues may be collected and homogenized, except that lung tissue can be prepared as a separate homogenate because MSD can be ideally reproduced from the lung homogenate. This lung homogenate represents the second serial passage of the original inoculum (MN 89-35477) in gnotobiotic pigs (Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990). Two filtrates can then

be prepared using .20 lm filter (Gelman Sciences, Ann Arbor, MI) and .10 lm filter (Millipore Corp., Bedford, MA). These filtrates can be aliquoted and stored at -70°C. All filtrates are free of bacteria and no viruses should be observed on direct electron microscopy using negative stained preparations.

Control inoculum. Homogenates of lung tissues prepared from 2 mock-infected gnotobiotic pigs can be used as inoculum in control pigs. This control inoculum can be prepared as 0.20 and 0.10 lm filtrates as described for the MSD inoculum.

Necropsy procedures and histopathology. Pigs can be euthanized 7 days after the original inoculation as previously described in Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990). Tissues can be collected, fixed in neutral buffered formalin, and processed for light microscopic examination as described in Collins et al., Am. J. Vet. Res., 50, 827-35 (1989). Specimens can be collected from turbinates, tonsil, trachea, brain, thymus, lung (apical, cardiac, diaphragmatic lobes), heart, kidney, spleen, liver, stomach, duodenum, jejunum, ileum, ascending and descending colon, blood and mesenteric lymph nodes. These tissues can be processed under microscopic examination to determine whether lymphomononuclear encephalitis, interstitial pneumonia, lymphoplasmacytic rhinitis, lymphomononuclear myocarditis or portal hepatitis is present. Lesions can be consistently observed in spontaneously infected pigs from herds with MSD inoculum (Collins et al., Minnesota Swine Conference for Veterinarians, Abstract, 254-55 (1990)). Fecal contents may also be collected and examined for virus particles as previously described in Ritchie et al., Arch. Gesante. Virus-forsche, 23, 292-98 (1968). Blood can be collected for immunologic assays

and tissues and cultured for bacteria as described in Example 3.

Discussion. Epidemiologic evidence supports the premise that MSD is an infectious disease, but no consensus agent has been isolated (Mengeling et al., In Proceedings of the Mystery Swine Disease Committee, 88-90 (1990)). Recently, the respiratory form of MSD was successfully transmitted to gnotobiotic pigs using .45 and .20 lm filtrates of an inoculum prepared from a pool of tissues from pigs with natural MSD infections (Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990) and Collins et. al., Minnesota Swine Conference for Veterinarians, Abstract, 254-55 (1990)). This suggests that the MSD agent(s) has an average diameter of 200 nm or smaller because the MSD symptoms re-occur in 3-day-old gnotobiotic pigs receiving the .20 lm filtrate. The experiment described below tests whether MSD is caused by a filterable agent(s) (with an average diameter of 200 nm or less) that can be transmitted via the respiratory tract to produce clinical disease and lesions in experimental pigs that resemble those observed in pigs naturally infected with MSD.

The experimental plan is designed to narrow the list of potential etiologic agents and use this information for isolation of the agent. Thus, in the absence of an in vitro system to study the etiology of MSD, an in vivo model may be used to further characterize the agent by various physical and chemical treatments of the inoculum followed by inoculation of gnotobiotic pigs to determine if the MSD agent(s) remain pathogenic (indicated by the presence of lesions).

Initially a filtration method can be used to determine the approximate size range of the MSD agent(s). The respiratory form of MSD can be transmitted using 0.20 lm filtrates. Bacteria, except

for mycoplasma (0.25-0.80 lm diameter), do not pass through filters of this size, and can be eliminated as primary agent(s). In addition, viruses (0.02-0.30 lm), chlamydia and rickettsia (0.30-0.50 lm) and naked nucleic acids (such as viroids) are filterable. A 0.10 lm filtrate can eliminate viruses with an average diameter greater than 0.10 lm, as well as mycoplasma, chlamydia and rickettsia, but not naked nucleic acids. Therefore, gnotobiotic pigs can be inoculated as follows: three pigs with a 0.20 lm filtrate of the MSD inoculum (positive controls); two with a 0.20 lm filtrate of the control inoculum (negative controls); three pigs with a 0.10 lm filtrate of the MSD inoculum and two pigs with similar filtrate of the control inoculum. Each group of pigs can be maintained in separate isolators and observed daily for clinical signs (anorexia, huddling, rough hair coats, diarrhea, rhinitis), euthanized at 7 days after the inoculation and tissues removed for histopathology as described above. Lesions typical of the respiratory form of MSD should appear in the pigs inoculated with the 0.20 lm filtrate as previously reported in Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990) and in Collins et al., Minnesota Swine Conference for Veterinarians, Abstract, 254-55 (1990), and in the 0.10 lm filtrate. No lesions should appear in pigs given the control inoculum.

Once the MSD agent is isolated, its chemical properties can be determined. Many microbial agents with high lipid concentrations in their outer walls or envelope (enveloped DNA and RNA viruses, mycoplasma, chlamydia and rickettsia) are inactivated by organic solvents as taught in Fenner et al., Veterinary Virology, Academic Press Inc., 21-38 (1987); Elisberg et al., Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, 5th Edition, American Public Health Association, 1061-108 (1979); and Schachter et

al., Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections, 5th Edition, American Public Health Association, 1021-59 (1979). The appropriate filtrate (0.20 or 0.10 lm) of the MSD and control inocula can be extracted twice with Genetron (1,1,2-trichloro-1,2,2-trifluoroethane) or chloroform to remove lipids. Two gnotobiotic pigs can be inoculated with the 0.20 lm filtrate (on Genetron treatment or chloroform) (positive controls), three pigs with Gentron/chloroform extracted filtrate of the MSD inoculum and two pigs with a similar preparation of the control inoculum. Pigs inoculated with a 0.20 lm filtrate of the control inoculum should not be used unless histopathology results suggest that this inoculum induces nonspecific lesions. If the MSD agent(s) contains an outer coat with a high lipid content (enveloped viruses, mycoplasma, chlamydia and rickettsia) then inactivation of infectivity by Genetron/chloroform, and the absence of lesions in the principals should occur.

Resistance of the MSD agent(s) to Genetron/chloroform extraction indicates the absence of lipid in the outer membranes of the agent(s) and suggests that it may have an outer protein coat. To evaluate this possibility, a Genetron/chloroform extracted filtrate can be treated with proteinase K (100 lg/ml) and 1% SDS at 37°C for 30 mins. to dissociate proteins. The inactivation of the MSD infectious agent by this treatment indicates that the infectious agent is possibly a naked virus or an unidentified microbial agent with a protein coat. Two pigs can be given the 0.20 lm untreated filtrate as a positive control inoculum; three pigs can receive the Genetron/chloroform extracted, proteinase K treated MSD filtrate and two pigs can be given a similar preparation of the control inoculum. If proteinase K digestion does not prevent transmission of the MSD agent, then this may indicate the possible presence of free RNA (viroid) or DNA in the

inoculum. This last possibility is unlikely, because such agents have not been confirmed as causes of diseases in animals (Diener, American Scientist, 71, 481-89 (1983)).

In summary, results from these experiments provide information on the relative size of the MSD agent and whether it contains a outer lipid membrane or protein coat necessary for infectivity. In addition, these experiments narrow the list of possibilities the infectious agent may be to either viruses, mycoplasma, chlamydia, rickettsia, naked nucleic acids, a new class of unidentified agents or a combination of any of the above.

## EXAMPLE 2

The purest form of an inoculum with the MSD infectious agent as determined from experiments in Example 1 may be used to transmit the MSD agent to pregnant sows to reproduce the reproductive form of the disease syndrome.

Discussion. Recently, transient anorexia and premature farrowing (both prominent clinical signs of MSD in the field) was induced in 2/2 sows inoculated with the same MSD inoculum, which produces respiratory lesions in gnotobiotic pigs. In addition, 15/29 (52%) of the pigs were stillborns and the remaining 14 pigs were weak and did not nurse well. No gross or microscopic lesions were observed in the stillborn pigs or the placenta and isolation procedures to detect microbial agents are now in progress. Therefore, the experiment described below tests whether the reproductive form of MSD can be transmitted to sows by intranasal inoculation and whether interference with fetal viability results from replication in maternal tissues but not fetuses.

Experiments in Example 1 will provide information on how the inoculum can be treated (filter size, organic solvent extraction and/or protease digestion) to provide the purest form of the MSD agent(s) for an inoculum. Because the MSD agent(s) has both a respiratory form in young pigs and a reproductive form in adult swine, it is necessary to reproduce the latter form of the syndrome to further verify that the infectious agent(s) is the putative cause of MSD. Field observations suggest that the MSD agent(s) can induce abortions in early, mid and late gestation (Mengeling et al., Proceedings of the Mystery Swine Disease Committee Meeting, 88-90 (1990)). Thus, a 90 day gestational sow is used as the experimental animal because it is possible to experimentally induce abortion in these animals inoculated with the MSD infectious agent.

Sows can be purchased from a commercial herd free of ongoing reproductive problems and MSD. Complete epidemiologic records on this herd can be computerized and information on gestation times, litter sizes, and average number of stillbirths can be made available for comparative studies. Groups of three sows each can be intranasally inoculated at 90 days of gestation with either the 0.20 lm filtrate (positive controls), a pathogenic but modified inoculum as dictated by results from Example 1, a 0.20 lm filtrate of the control inoculum (negative control), and a control inoculum modified as indicated by results of experiments in Example 1. Each group of sows can be housed in separate isolation rooms and examined daily until gestation is complete. Temperatures and clinical signs (anorexia, respiratory problems such as coughing, sneezing, panting, and increased respiration) can be noted daily. Sampling of sows can be restricted to a pre- and post-farrowing blood sample for serology. The actual date of farrowing can be noted and the number of stillborns, mummified fetuses, live "weak" pigs and live "normal"

pigs determined. Fetuses can be examined for gross and microscopic lesions as described in Example 1 and fetal tissues processed for microbiologic assays as described in Example 3. The fetal sera can also be assayed for the presence of gammaglobulins and antibodies to PPV and EMCV (Joo et al., In Proceedings of the Mystery Swine Disease Committee Meeting, 62-66 (1990) and Kim et al., J. Vet. Diagn. Invest., 1, 101-4 (1990)). Pigs born live can be observed for one week and morbidity and mortality recorded, after which these pigs can be euthanized and the tissues collected for light microscopic and microbiologic examination as described for the fetuses.

The 0.2 lm and the modified filtrates of the MSD inoculum are pathogenic for sows and induce anorexia, possibly a mild fever and premature farrowing with a large number of stillborn and weak pigs in each litter. This illustrates evidence that the inoculum contains the MSD agent(s). Sows inoculated with the control inoculum farrow near term and have litter sizes within the normal range for the herd of origin as determined from the available epidemiologic database on this herd. No lesions in the stillborn pigs are found and a high rate of mortality among the surviving weak pigs within one week after birth is observed.

## EXAMPLE 3

Tissue samples collected from gnotobiotic piglets inoculated with the MSD inoculum and euthanized at various times post-inoculation can be used to isolate and identify the MSD agent(s), to determine the sequential development of lesions, and to ascertain whether the MSD agent(s) is immunosuppressive.

Bacteriologic assays. Tissues are only cultured to verify sterility because MSD can be transmitted to pigs using .20 lm filtrates (Benfield et

al., Am J. Vet. Res., 49, 330-36 (1988)). Lung homogenates can be cultured for mycoplasma and frozen sections of the lung can be examined for mycoplasmal antigens as described in Ross, Diseases in Swine, Iowa State University Press, 469-83 (1986).

Virologic assays. Tissues (turbinates, lung, heart, liver and brain) in which lesions were observed in a previous study are used (Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990)). Tissues from principal and control pigs are treated similarly in all procedures. Results from preliminary animal transmission studies (Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990)) indicate severe lesions and cell destruction in the turbinates and lungs of gnotobiotic pigs. In addition, there are large numbers of macrophages and monocytes in the lungs of the pigs inoculated with the MSD agent(s). Primary porcine turbinates, lung cells and alveolar macrophage cultures can be developed from 3-day-old gnotobiotic pigs for use in the virus isolation studies. Alveolar macrophage cultures can be prepared and maintained as described (William, 1978). In addition, primary pig kidney and thyroid cells, swine testis cells, and can be used for EMCV detection baby hamster kidney cells (Kim et al., J. Vet. Diagn. Invest., 1, 101-04 (1989) and Joo et al., In Proceedings of the Mystery Swine Disease Committee Meeting, 62-66 (1990)).

Turbinates and lung can be prepared as separate 10% (weight/volume) homogenates, whereas heart, liver and brain should be pooled. Hanks balanced salt solution can be used as the diluent, but antibiotics can be omitted since these tissue suspensions can also be used for detection of chlamydia or rickettsia, which are sensitive to some antibiotics. Approximately 0.5 ml of

each homogenate can be adsorbed on sixteen replicates of each cell type. Eight replicates can be incubated at 35°C: four replicates on roller tubes (two replicates containing media with 5 lg/ml trypsin and two without trypsin) and four replicates on stationary cultures (two with and two without media containing trypsin). A similar set of eight replicates can be maintained at 37°C. Cultures should be examined daily for cytopathic effects (CPE) and if negative, blind passaged at weekly intervals for 5 passages. At each passage, freeze-thaw lysates of the cultures should be examined by electron microscopy for viral particles and scrapings of cells used in an immunofluorescence assay for viral antigens (Benfield et al., Arch. Virol., 82, 195-209 (1984)).

The above tissues can also be examined for cell-associated viruses by either cocultivation of 2 $mm^2$ cubes of tissue or trypsinized cells ($2X10^6$) with each of the above cell cultures seeded in 6-well plastic plates. Tissue pieces should remain in contact with cells for 7 days and then removed. These procedures are similar to those that have been previously used for the isolation of cell-associated herpes viruses in Benfield et al., Arch. Virol., 82, 195-209 (1984). Cultures should be examined daily for cytopathic effects for 35 days with subpassages (trypsinized cells) at 7 day intervals before being discarded as negative. At each subpassage a portion of the cell culture can be used for negative staining electron microscopy and immunofluorescence to check for viral particles or antigens as described in Benfield et al., J. Clin. Microbiol., 16, 186-90 (1982).

Immunofluorescence assays on frozen tissues and inoculated cell cultures. Immunofluorescence assays on frozen tissues and cell scrapings can be done as previously described in Benfield et al., Arch. Virol., 82, 195-209 (1984) and Benfield et al., Am. J. Vet. Res., 49, 330-36 (1988). The frozen tissues and cell

scraping can be screened for PPV, EMCV (See Example 4 for definition of acronyms) and the MSD agent(s). Conjugates for PPV and EMCV are available at the South Dakota Animal Disease Research and Diagnostic Laboratory. A hyperimmune sera in gnotobiotic pigs can be prepared from the purest form of the MSD inoculum. Two pigs can be inoculated intranasally, and then given a subcutaneous booster of the MSD inoculum in Freund's incomplete adjuvant at 2 and 4 weeks after the initial inoculation. Sera can be harvested from this pig 2 weeks after the last booster. A control sera is also prepared in two gnotobiotic pig using the control inoculum and the same immunization protocol described for the MSD inoculum. These sera can be used as primary antibody and goat or rabbit anti-porcine immunoglobulin conjugated with fluorescein isothiocyanate as secondary antibody to detect MSD antigens in frozen tissue sections and cell cultures.

Assays for chlamydia and rickettsia. Procedures for the detection of chlamydia (Storz, Laboratory Diagnosis of Livestock Abortion, 3rd Edition, Iowa State University Press, 37-48 (1990)) and rickettsia (Zeman et al., Laboratory Diagnosis of Livestock Abortion, 3rd Edition, Iowa State University Press, 175-185 (1990)) in tissue homogenates are currently used in the South Dakota Animal Disease Research and Diagnostic Laboratory.

Serologic assays. Sera collected from control and inoculated pigs can be assayed for the presence or absence of antibody to PPV and SIV (hemagglutination inhibition), Leptospira (micro-agglutination), and EMCV (viral neutralization) (see Example 4 for definition of acronyms). Previous results indicated that serology to other common microbial agents were negative (Collins, et al., 71st Meeting of the Conference of Research Workers

in Animal Disease, Abstract No. 2 (1990)) and need not be repeated here.

Immunologic assays. Tissues and blood can be collected from MSD inoculated and control pigs so that their immunological status can be determined. Porcine leukocytes can be isolated from peripheral blood by single step discontinuous gradient floatation on Histopaque 1077 as taught by Pescovitz et al., J. Immunol., 134, 37-44 (1985). Cells from lymph nodes, spleen and thymus can be spilled into single cells by moderate mincing of the tissues and collection of the resultant cell suspensions (Hurley et al., Cancer Res., 47, 3729-35 (1987)).

Porcine leukocyte phenotypes can be determined using a panel of monoclonal antibodies available through the American Type Culture Collection and Joan Lunney (USDA Beltsville). These include antibodies for the measurement of total T cells, pCD2 (MSA4; Hammerberg et al., Vet. Immunol. Immunopathol., 11, 107-21 (1986), helper/class II MHC dependent T cells, pCD4 (74-12-4; Lunney et al., Vet. Immunol. Immunopathol., 17, 135-144 (1987), cytotoxic-suppressor/class I MHC dependent T cells, pCD8 (74-2-11; Ibid), macrophages and granulocytes (74-22-15A; Ibid), thymocytes and peripheral B cells, pCD1 (76-7-4; Ibid), and pig MHC class II antigens equivalent to human DRw (MSA3; Hammerberg et al., Vet. Immunol. Immunopathol., 11, 107-21 (1986)) and DQw (TH21A and others (VRMD, Pullman, WA; Davis et al., Hybridoma Technology in Agriculture and Veterinary Research, Rowman and Allanheld, 121-50 (1984)). Isotype specific monoclonal antibodies to porcine immunoglobulins are also available (Paul et al., J. Vet. Res., 50, 471-79 (1989)), and can be used at twice minimum saturating concentration for indirect fluorescent staining of leukocytes from peripheral blood, lymph node, and Peyer's patches (Hurley et al.,

Vet. Immunol. Immunopathol., 25, 177-93 (1990)). To achieve two color analysis, cells can also be stained with rPE labeled avidin after being tagged with biotin-bound (Pierce kit #21333) antibodies. Cells can be analyzed by flow cytometry or a two color analytical fluorescent microscope (PTI FSCAN system). Co-detection in the 488 nm laser line on the flow cytometer or using the dual analytical fluorescent microscope can easily be attained. Intensity of cellular fluorescence and percentage of positive cells can also be determined.

In vitro functional assays such as lectin mitogenesis with concanavalin A, pokeweed mitogen (PWM) and phytohaemagglutinin (PHA), can be performed as described in Hammerberg et al., Am. J. Vet. Res., 50, 868-74 (1989). Antigen specific in vitro T cell responses to lysozyme can also be modeled after their technique. B cell proliferative assays can be performed with E. coli and S. typhimurium LPS or anti-immunoglobulin as reported in Symons et al., Int. Archs. Allergy Appl. Immun., 54, 67-77 (1977). In vitro antibody production, induced with PWM can be accomplished and quantitated as described in Hammerburg et al., Am. J. Vet. Res., 50, 868-74 (1989). Macrophage production of IL-1 after 48 hour exposure to E. coli LPS can be measured in the mouse thymocyte assay (Mizel, Immunological Rev., 63, 51-72 (1982)). IL-2 production by PHA stimulated lymphocytes can be measured as described by Stott et al., Vet. Immunol. Immunopathol., 13, 31-38 (1986). An isotype-specific anti-lysozyme ELISA can be done utilizing the monoclonal antibodies to porcine immunoglobulin isotypes (Paul et al., Am. J. Vet. Res., 50, 471-79 (1989)).

To assess in vivo antibody production three piglets inoculated with MSD and three inoculated with control inoculum can be injected with a 2% suspension of sheep erythrocytes and a 10 lg/ml solution of bovine serum albumin at separate sites at 5, 7, 10, 14, and 24

days after their original inoculation. Pigs are euthanized at 24 days after the original inoculation, and tissues are collected for histopathology as described in Example 1 and blood collected to assay for antibody. The total antibody level and the specific IgG and IgM responses to each antigen can be measured by antigen specific radial immunodiffusion or ELISA. Antigen specific plaque assays can be performed on spleen cells to assess the frequency of B cell clones in the infected and control animals (Kappler, J. Immunol., 112, 1271-85 (1974)).

Discussion. In previous studies, pigs were euthanized at 7, 8 and 25 days after the inoculation to demonstrate the presence of lesions. Tissues removed from these pigs were subsequently used in assays for microbiologic agents, but isolation attempts were negative (Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990). Tissues from stillborn fetuses have also been used without success. These results suggest that the MSD agent(s) is either not present or present in minute quantities in fetal tissues and tissues collected at 7 days after the inoculation. In addition, the primary pig kidney cells used for isolation may not be permissive for the MSD agent. Therefore, the experiment described below tests whether successful isolation of the MSD agent may depend on the collection of tissues at earlier times after inoculation, when the agent is replicating and high titers of the agent are present in target tissues such as the lung. Based on the results obtained from experiments in Example 1, there will be some indication as to the properties of the infectious agent that causes MSD. These results can be depended upon to determine which microbiologic procedures to use for isolation and identification of the MSD agent.

Pigs can be sacrificed at various intervals after inoculation. A variety of tissues can be collected and a variety of cell types used to improve the chances of isolating the virus. Tissues collected from experimentally inoculated gnotobiotic pigs can be used because tissues collected from these pigs transmit the respiratory form of MSD. Thus, there is a high probability that these tissues contain the MSD agent(s). The virus isolation procedure includes methods to accommodate temperature sensitive viruses (incubation at 35°C), viruses (such as reoviruses, enteroviruses) that are activated by proteases and cell-associated viruses (herpes viruses, paramyxoviruses, retroviruses).

Five gnotobiotic piglets (3 inoculated intranasally with the purest form of MSD and 2 with control) can be euthanized at 1, 3, 5, 7, 10 and 14 days after being inoculated. Necropsy procedures and tissues to be removed for histopathology should be followed pursuant to the experimental design disclosed in Example 1. Tissues for virus isolation should only be collected from pigs euthanized at 1, 3, 5 and 7 days after inoculation, since previous attempts to identify the MSD agent(s) with tissue from pigs sacrificed at 7 days after inoculation are usually unsuccessful. Pigs euthanized at 10 and 14 days can be used as a source of tissues for histopathology to monitor progression of the lesions and in the immunologic assays.

By sacrificing the pigs at sequential time intervals after inoculation, the development of lesions may be examined. The results of these studies can provide information on tissue tropism (lesions present in tissues with replication) and how rapidly lesions develop. Early destruction of the turbinates will occur followed at later times by the development of interstitial pneumonia in the lungs, inflammation in the heart, liver and brain. Lesions can be identified and graded in the various tissues as 0 (no lesions), 1

(mild), 2 (moderate) and 3 (severe) at each time interval after inoculation.

The high rate of isolation of secondary pathogens from pigs with the respiratory form of MSD indicates that these pigs may be immunosuppressed (Keffaber, AASP Newsletter, 2, 1-10 (1990)), and the preliminary data (see Table in Addendum) indicates depressed responsiveness of various lymphocytes isolated from pigs inoculated with the MSD agent. However, this study was done with a limited number animals. It is expected that the immunologic assays will permit a determination of whether the MSD agent(s) specifically suppress a specific subpopulation of either T or B lymphocytes and whether this suppression is expressed functionally in the in vitro and in vivo assays.

## EXAMPLE 4

Objective. The goal of this experiment is to prepare hyperimmune antisera in a gnotobiotic pig to an isolate of MSD for use as a diagnostic reagent and for further characterization of the antigenic properties of MSD.

Background. Previous studies done in gnotobiotic pigs at South Dakota State University in collaboration with the University of Minnesota indicated that pooled tissue homogenates from field case MN 89-35477 induced lung lesions in gnotobiotic pigs. Pooled tissue homogenates from these pigs have subsequently been used to produce clinical disease and respiratory lesions characteristic of MSD in 3-day-old gnotobiotic pigs (Collins et al., 71st Meeting of the Conference of Research Workers in Animal Disease, Abstract No. 2 (1990) and Collins et al., Minnesota Swine Conference for Veterinarians, Abstract, 254-55 (1990)). Lung and tissue homogenates were prepared from this second passage of the original inocula in gnotobiotic pig (90 X

75) to produce a second inocula. The second inocula can be used as inocula and antigen to produce the hyperimmune sera in this experiment.

Procedure to Accomplish the Objective

Gnotobiotic pigs. Gnotobiotic pigs can be derived and maintained as previously described in Benfield et al., Am. J. Vet. Res., 49, 330-36 (1988) and and Collins et al., Am. J. Vet. Res., 50, 827-835 (1989).

Inoculation of hyperimmune sera. Hyperimmune sera can be prepared by initially inoculating one gnotobiotic pig as described above. Pigs can then be given a booster consisting of 1 ml of the second inocula and 1 ml of Freund's Incomplete Adjuvant at 14 and 21 days after the original inoculation (Harlow and Lane, 1988). The pig should then be killed and exsanguinated 10 or 14 days after the last inoculation. Sera should be harvested and dispensed into appropriate aliquots and frozen at -20°C.

Serology. The hyperimmune sera can be tested for the presence of antibodies to common pathogens of swine as commonly done in most diagnostic laboratories. This sera can be tested for antibodies to Hemophilus, Brucellosis, Leptospira (6 serovars), pseudorabies (PRV), parvovirus (PPV), encephalomyocarditis virus (EMC), and Swine Influenza Virus (SI).

Results. The pig used for preparation of the antisera was pig # 4B (experimental number 90 X 238). This pig was inoculated on 11/1/90 and observed daily for clinical signs until killed on 11/29/90. Clinical signs are summarized in Table 1. Unfortunately the continuing degenerate condition of the pig mandated that it be euthanized after only one booster on 11/13/90.

The pig was euthanized 16 days after the initial booster on 11/29/90.

Serology results were negative for all the above agents except PPV, which had a titer of 16, 384 (See Table 2). A pretitered sera on this pig was not conducted.

Samples of lung, heart, brain, kidney, colon, small intestine, turbinates, spleen, stomach and trachea were collected when the pig was necropsied. These samples were evaluated and it was found that the lungs from this pig had lesions of severe pneumonia typical of that seen with field cases of MSD.

The results of this experiment confirm initial studies that an infectious agent is present in the second inocula because it can induce clinical disease and lesions typical of those observed in natural cases of MSD.

Table 1

| Date | Observations of Inoculated Piglet |
|---|---|
| 10/29 | Surgery |
| 11/1 | Pig inoculated i.n. with 0.5 ml of above inocula using Nebulizer at 5:00 p.m. |
| 11/2 | Not observed. |
| 11/3 | This pig has 2 times as much milk in bowl as the control pigs. I'm not sure how well the pigs are eating or if the agent is the cause of the anorexia. Feces normal. |
| 11/4 | May be a little slow but alert and strong, 1/2 milk left, feces soft and brown. |
| 11/5 | 9 a.m.: strong, alert, drank most of milk, feces mucoid and brown (2).<br>4 p.m.: strong, alert, drank most of milk, feces mucoid and brown (2). |
| 11/6 | 9 a.m.: strong, alert, drank 1/2 of milk, feces brown mucoid (2).<br>6 p.m.: strong, alert, 1/2 of milk left, feces loose yellow brown (2). |
| 11/7 | 9 a.m.: strong, alert, drank most of milk, feces light brown mucoid (2).<br>3 p.m.: strong, alert, drank most of milk, feces light brown mucoid (2). |
| 11/8 | 2 p.m.: Alert, does not drink milk as well as controls, slower than controls, mucoid yellow feces. |
| 11/9 | 8 a.m.: Alert, still does not drink as aggressively as controls, pasty feces.<br>5 p.m.: same observation as 8 a.m. |

Table 1 Cont'd

| | |
|---|---|
| 11/10 | 6 p.m.: alert, vigorous, rubs snout aggressively against feed pan, feces brown loose, not eating like controls. |
| 11/11 | 6 p.m.: alert, vigorous, rubs snout vigorously against feed pan, feces pasty, not eating as well as controls, still milk in pan, by 6:30 p.m. controls had finished eating. |
| 11/12 | 9 a.m.: alert, but not as aggressive as controls, after 5 minutes the controls have cleaned pans but this pig still has at least 2/3 of milk in bowl. Some snout rubbing. |
| 11/13 | Inoculated with 90 X 75 lg & pool using nebulizer (.5 m) and sp. IFA (1 ml) at 4 p.m. Alert but not as aggressive as controls, still has 1/2 pan of milk but controls have consumed all their milk. No snout rubbing, feces pasty. |
| 11/15 | 7 p.m.: alert but not as aggressive as controls, still east slow, rough hair coat, but gaining weight like controls. |
| 11/16 to 11/24 | Not much change, alert but steady declining in activity |
| | 1 p.m. (11/24): respiration seems to be more rapid, hair coat is rough, not gaining weight like controls. |
| 11/29 | Euthanized - blood collected for H.I. sera. Usual times collected for histopathology including tonsil. Blood collected for lymphocyte mitogenic assays. No gross lesions noted. Set up turbinate explant cultures. |

Table 2

Antibody Tests of Inoculated Piglet

1. Swine influenza - negative
2. Swine Encephalomyocarditis - negative
3. Swine APP - negative
4. Swine PRV - negative
5. Swine PPV - negative
6. Swine Brucella - negative
7. Swine Leptospirosa - negative
8. Swine EPI - negative

EXAMPLE 5

Three pilot studies are described. A gnotobiotic pig study was undertaken to show that field material could be used to infect and cause the respiratory component of the syndrome in germ-free pigs. A second study using conventional weaned pigs was undertaken to determine if the respiratory disease seen in gnotobiotic pigs could be reproduced in conventional pigs. And finally, a pregnant sow study was undertaken to determine if the reproductive failure component of the syndrome could be experimentally reproduced.

Material and Methods

Field case. A 160-sow farrow-to-finish herd in West Central Minnesota experienced an outbreak of MSD with typical MSD symptoms. A live sow, live neonatal piglets and stillborn fetuses were submitted to the Minnesota Veterinary Diagnostic Laboratory for examination including gross necropsy, histopathology and routine microbial investigation. An inoculum was prepared for experimental use with several tissues from clinically ill neonatal pigs.

Gnotobiotic study. Six hysterectomy-derived gnotobiotic piglets were inoculated intranasally at 3 days of age with the field inoculum (10% homogenates, various tissues). Filtered (0.22 lm) and unfiltered inoculum were used. Two control piglets were inoculated with media only. Clinical signs were monitored daily and the pigs were euthanized 8 days post-inoculation, except one animal which was held for the production of hyperimmune serum. Tissues for virus isolation and histologic examination were collected at necropsy along with sera which was screened for antibodies to leptospira, chlamydia, eperythrozoon, Aujeszky's disease virus, porcine parvovirus, encephalomyocarditis virus, hemagglutinating encephalitis virus, swine influenza virus, bovine respiratory syncytial virus, canine distemper virus, bovine viral diarrhea and hog cholera. The original inoculum and tissues from gnotobiotic piglets were inoculated onto continuous and primary cell lines for 3 passages. In addition, direct and immunoelectron microscopy was performed.

Conventional pig study. Three conventional 28-day-old weaned pigs from a farm with no history of MSD were intranasally inoculated with 10% lung homogenates from affected gnotobiotic piglets. A lung homogenate from a negative gnotobiotic pig was used as inoculum for a control. Piglets were monitored daily for clinical signs and were necropsied 8 days post-inoculation. Sera/tissues were processed as described above.

Pregnant sow study. Eight multiparous sows with known historical due dates from a farm free of MSD were used in this study. Three weeks prior to the expected farrowing date, six sows were intranasally inoculated with affected gnotobiotic lung homogenates and two sows with negative lung homogenates. Clinical

signs were monitored daily. The sows were allowed to farrow naturally and when possible the farrowings were attended in order to collect presuckled sera from live born pigs. Sows and live pigs were euthanized shortly after farrowing and tissues were collected for histopathology and virus isolation. Sera or fetal thoracic fluids were also collected.

**Results**

**Field study.** No gross lesions were seen at necropsy. Microscopic examination of nursing piglets revealed necrotizing interstitial pneumonia and lymphomononuclear encephalitis. The fetuses did not have lesions but the sow did have a mild encephalitis. Microbiologic examination did not yield conclusive results.

**Gnotobiotic study.** The piglets became anorexic and developed rough hair coats 3 days post-inoculation. Controls remained normal. Microscopic lesions were found in the principals inoculated with filtered or non-filtered material. The lesions were similar to field cases and included: necrotizing interstitial pneumonia (6/6 [six out of six piglets]), lymphoplasmacytic rhinitis (4/6), lymphomononuclear encephalitis (2/6) and myocarditis (1/6). No etiologic agent was identified either by pre- and post-inoculation serology or through inoculum/tissue examination.

**Conventional weaned pig study.** Clinically, the principals became dull and anorexic 2 days post inoculation. The animals appeared chilled even though adequate heat was provided. One pig had an elevated temperature (41.5°C) 6 days post-inoculation. Interstitial pneumonia, encephalitis and myocarditis were found in the principals but not the control.

Pregnant sow study. Clinically, only 2 principals showed any significant temperature rises (1.5°C) day 3 or 5 post-inocluation. However, anorexia was noted in 4/6 sows at day 4 or 5 post-inoculation. Three sows farrowed up to 7 days early and three sows farrowed on time. Over 50% of the fetuses from infected sows were born dead while the controls had normal litters. Both stillborns and late-term mummies were found in infected litters. Laboratory findings were not conclusive--no specific agent has been identified and no lesions have been noted in fetuses to date.

Although no causative microorganism has been identified, the findings suggest MSD can be transmitted experimentally to gnotobiotic and conventional pigs (using field tissues from one farm). Both the respiratory and reproductive forms of MSD were reproduced. The agent involved appears infectious, filterable at .22 um and is seemingly fastidious.

Discussion

MSD is an important emerging disease not only in the United States but also throughout the world. In order to study the disease in a controlled setting, gnotobiotic pigs were inoculated intranasally at 3 days of age with tissue homogenates from a farm experiencing the clinical signs of MSD. Microscopic lesions similar to field cases including necrotizing interstitial pneumonia and to a lesser extent lymphoplasmacytic rhinitis, lymphomononuclear encephalitis or myocarditis were seen in principals but not controls. Using lung homogenates from the gnotobiotic pigs, intranasal inoculation of conventional 4-week-old weaned pigs produced similar lesions. Multiparous pregnant sows were also inoculated with gnotobiotic lung homogenates 3 weeks prior to their due dates. Clinically these sows went through a period of anorexia and farrowed up to 7 days early. Over 50% of the fetuses were either

stillborn or in the beginning stages of mummification. Although no causative microorganism has been identified, the findings indicated the disease can be isolated and transmitted experimentally with field tissues to gnotobiotic pigs and from gnotobiotic pigs to conventional weaned pigs or pregnant sows. This study provides a model for both the respiratory and reproductive forms of the disease which will lead to further investigations of the pathogenesis and diagnosis of MSD.

WHAT IS CLAIMED IS:

1. A vaccine suitable for use in prevention of mystery swine disease, comprising:

an inactivated or attenuated infectious agent derived from swine tissue infected with mystery swine disease in combination with a pharmaceutical carrier.

2. A vaccine according to claim 1 wherein the infectious agent is obtained from an inoculum of a filtered homogenate of swine lung tissue infected with mystery swine disease.

3. A vaccine according to claim 2 wherein the homogenate has been purified by neutralization with antibody sera to swine diseases selected from the group consisting of hemophilus, brucellosis, leptospire, parovirus, pseudorabies, encephalomyocarditis, enterovirus, swine influenza and any combination thereof.

4. A vaccine according to claim 1 wherein the infectious agent is obtained from the cell culture medium of <u>in vitro</u> cultured mammalian organ cells treated with an inoculum obtained from the swine lung tissue infected with mystery swine disease.

5. A vaccine according to claim 2 wherein the filtered homogenate contains biological particles having a size of no greater than 1.0 micron.

6. A vaccine according to claim 5 wherein the size is no greater than 0.5 micron.

7. A vaccine according to claim 1 wherein the infectious agent is a virus.

8. A vaccine according to claim 7 wherein the virus has been purified by regressive cell culturation.

9. A vaccine according to claim 1 wherein the swine tissue is swine lung tissue which is infected with mystery swine disease and exhibits th8ickened alveolar septae.

10. A method of making a MSD vaccine containing an inactivated or attenuated MSD infectious agent, the method comprising:

identifying swine lung tissue having thickened aveolar septae, degenerating cells and debris in aveolar spaces;

homogenizing the swine lung tissue with a pharmaceutically acceptable aqueous solution to form a mixture;

filtering the mixture through a series of filters to produce a filtered homogenate containing the MSD infectious agent; and

inactivating or attenuating the MSD infectious agent in the filtered homogenate to produce the MSD vaccine.

11. A method according to claim 10, wherein the homogenate is filtered to contain particles having a size no greater than about 1.0 micron.

12. A method according to claim 11 wherein the size is no greater than about 0.5 microns.

13. A serum suitable for treatment of swine infected with mystery swine disease, comprising:

the semi-purified blood serum of a mammal inoculated with an infectious agent derived from swine tissue infected with mystery swine disease.

14. A serum according to claim 13 wherein the infectious agent is obtained from an inoculum of a filtered homogenate of swine lung tissue infected with mystery swine disease.

15. A serum according to claim 13 wherein the infectious agent is obtained from the cell culture medium of in vitro cultured mammalian organ cells treated with an inoculum obtained from swine lung tissue infected with mystery swine disease.

16. A method for diagnosis of mystery swine disease in swine, comprising:

obtaining a lung tissue sample from the swine,

forming a liquid homogenate of the sample,

adding the liquid homogenate to a vessel for immobilizing a viral material to form an immobilized mixture,

adding to the immobilized mixture a nonswine, mammalian species antibody serum to mystery swine disease to form a complex,

adding a labeled anti-species antibody to detect the complex.

17. A method according to claim 16 wherein the nonswine mammalian species antibody is a monoclonal antibody.

18. A method according to claim 16 wherein the labeled anti-species antibody carries a radioactive or color producing enzyme label.

19. A method for diagnosis of mystery swine disease in swine, comprising:

forming a liquid homogenate of a lung tissue sample from the swine,

adding to the liquid homogenate a precipitating antibody which is immunospecific for mystery swine disease virus,

detecting the presence of a precipitate of an antibody-antigen complex.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/07826

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N7/00; C07K15/00; G01N33/569; A61K39/12
A61K39/42

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C12N ; C07K ; G01N ; A61K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| Y | PHIND Database,PJB Publications Ltd.,Surrey ,GB<br>Abstract number 00278268<br>'Dutch team isolates mystery pig ...'<br>& Animal-Pharm 230,21-06-91 p21 | 1,13 |
| A | | 8,9,10, 16 |
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| Y | DATABASE WPIL<br>Week 8741,<br>Derwent Publications Ltd., London, GB;<br>AN 87-286929[41]<br>& JP,A,62 198 626 (ZH BISEIBUTSU KAGAKU KEN) 2 September 1987<br>see abstract | 1 |
| A | | 4,15 |
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| | -/-- | |

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 19 JANUARY 1993 | 25. 02. 93 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | GURDJIAN D. |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9207826
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This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
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| | | JP-T- | 2503865 | 15-11-90 |

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82